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Increased regulatory and decreased CD8+ cytotoxic T cells in the blood of patients with idiopathic pulmonary arterial hypertension

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Abstract: **BACKGROUND:** An association between pulmonary arterial hypertension (PAH) and various immune disorders is well established. Recently, the role of an intact immune system in protecting against pulmonary angioproliferation was shown in an animal model. **OBJECTIVE:** To elucidate the role of T cells in human PAH, we comparatively studied T cell subclasses with emphasis on regulatory T cells (T(reg)) in the peripheral blood of patients with idiopathic pulmonary arterial hypertension (IPAH) and healthy controls. **METHODS:** Isolated peripheral blood mononuclear cells from 36 patients diagnosed with IPAH and 33 healthy controls were stained with fluorescently labeled monoclonal antibodies against superficial T cell markers (CD3, CD4, CD8, CD25) and FoxP3, the intracellular marker of T(reg) cells. The relative cell distribution was analyzed by flow cytometry. The functionality of patient and control T(reg) cells was assessed by coculture of T(reg) with nonregulatory T cells from the same individual. **RESULTS:** Significantly less CD8+ T cells ($p = 0.02$) and more CD25hi+ and FoxP3+CD4+ T cells were found in the peripheral blood of patients compared with controls ($p = 0.009$ and $p < 0.001$, respectively). The percentage of FoxP3+ cells within the CD25hi+CD4+ T(reg) cells was similar. T(reg) cell functionality was equal in patients and controls. **CONCLUSION:** Our findings of decreased CD8+ T cells and increased T(reg) cells in the peripheral blood of patients with IPAH are novel and may have implications for directing future research in the field to elucidate the differential role of T cells and the immune system in IPAH. 2007 S. Karger AG, Basel

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Increased Regulatory and Decreased CD8+ Cytotoxic T Cells in the Blood of Patients with Idiopathic Pulmonary Arterial Hypertension

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For editorial comment see p. 253

Key Words

CD4 · CD8 · CD25 · FoxP3 · Idiopathic pulmonary arterial hypertension · Regulatory T cells

Abstract

Background: An association between pulmonary arterial hypertension (PAH) and various immune disorders is well established. Recently, the role of an intact immune system in protecting against pulmonary angioproliferation was shown in an animal model. **Objective:** To elucidate the role of T cells in human PAH, we comparatively studied T cell subclasses with emphasis on regulatory T cells (T_{reg}) in the peripheral blood of patients with idiopathic pulmonary arterial hypertension (IPAH) and healthy controls. **Methods:** Isolated peripheral blood mononuclear cells from 36 patients diagnosed with IPAH and 33 healthy controls were stained with fluorescently labeled monoclonal antibodies against superficial T cell markers (CD3, CD4, CD8, CD25) and FoxP3, the intracellular marker of T_{reg} cells. The relative cell distribution was analyzed by flow cytometry. The functionality of patient and control T_{reg} cells was assessed by coculture of T_{reg} with nonregulatory T cells from the same individual. **Results:** Significantly less CD8+ T cells ($p = 0.02$) and more CD25hi+ and FoxP3+CD4+ T cells were found in the peripheral blood of patients compared with controls ($p = 0.009$ and $p < 0.001$, respectively). The percentage of FoxP3+ cells within the CD25hi+CD4+ T_{reg}

cells was similar. T_{reg} cell functionality was equal in patients and controls. **Conclusion:** Our findings of decreased CD8+ T cells and increased T_{reg} cells in the peripheral blood of patients with IPAH are novel and may have implications for directing future research in the field to elucidate the differential role of T cells and the immune system in IPAH.

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Introduction

Idiopathic pulmonary arterial hypertension (IPAH) is a progressive disease with a dismal prognosis ultimately leading to right ventricular failure and death. Lumen obliteration of the microscopically fine precapillary arterioles due to endothelial proliferation and formation of complex vascular lesions are important aspects of the pathobiology [1–3]. Although germline mutations of the gene encoding bone morphogenetic protein receptor II (BMPRII), a member of the transforming growth factor superfamily, have been identified in some patients with familial and sporadic IPAH [4–7], how germline BMPRII mutations promote the development of PAH remains unclear. Thus, other mechanisms contributing to the pathogenesis of PAH are of great interest and currently under investigation. One controller of pulmonary hypertension may be the immune system, based on the following facts:

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PAH is associated with a number of collagen vascular autoimmune disorders [8–14] (where BMPRII mutations are not prevalent) [15]; secondly, patients with PAH often have antinuclear, antiphospholipid or other autoantibodies [16–20]; plexiform lesions are surrounded by immune cells [3, 21–23], and alterations in blood cytokines have been reported [24]. PAH also occurs in association with HIV infection, where CD4 lymphocytes and regulatory T cells are depleted [25]. Recently, we showed that athymic rats, which lack T lymphocytes, develop severe angioproliferative PAH when exposed to the vascular endothelial growth factor inhibitor SU5416 in a normoxic environment [26–28]. If the immune system of these athymic rats was reconstituted by injecting splenocytes from euthymic syngeneic animals before administration of SU5416, the animals were protected from the development of exuberant pulmonary vascular lesions [27]. Hence, an intact immune system and especially the T cell compartment seem to play a role in the development of PAH.

How the immune system discriminates between self and non-self and establishes and maintains unresponsiveness to self has been a key issue in immunology since the proposition of the clonal selection theory [29]. The contribution of regulatory (or suppressor) T cells (T_{reg}) or even their existence as a cellular entity has been controversial until recently, mainly because of the lack of a reliable marker to identify them and the ambiguity of their functions at the molecular level [30]. Research in the last years has discovered several cell surface markers that could operationally differentiate T_{reg} from other T cells [31, 32]. Recently, FoxP3, a transcription factor of the forkhead/winged-helix family, has been identified as a very specific marker of T_{reg} cells [33–36]. FoxP3+ T_{reg} cells have been shown to be essential for the induction and maintenance of self-tolerance and prevention of T-cell-mediated autoimmunity [36–38]. In healthy humans, this population accounts for 5–10% of the peripheral CD4+ T cells. Alterations in the number of T_{reg} cells in the peripheral blood have been reported in various immune disorders, some of them associated with PAH, and cancer [39–45]. Whereas T_{reg} cells were found to be qualitatively and/or quantitatively deficient in many autoimmune diseases (e.g., multiple sclerosis, graft versus host disease, systemic lupus erythematosus, type I diabetes or rheumatoid arthritis) [46–49], they have been shown to be increased in some tumors and infections [41, 42, 45, 46, 50, 51]. The aim of the present study was to investigate peripheral blood T lymphocyte subsets with emphasis on T_{reg} cells in IPAH compared with healthy controls in the hope of elucidating one step toward a better understanding of the role of the immune system in human PAH.

Material and Methods

Study Design and Subjects

The present study is a prospective clinical investigation on lymphocyte subsets in the peripheral blood of patients compared with healthy controls. Patients were diagnosed with IPAH according to the WHO classification [52] if the mean pulmonary artery pressure was ≥ 25 mm Hg along with a pulmonary arterial occlusion pressure ≤ 15 mm Hg assessed by right heart catheterization, and if an extensive clinical work-up did not reveal other conditions responsible for pulmonary hypertension [52]. Healthy volunteers working at the University of Colorado Health Sciences Center served as controls. The study was approved by the local institutional review board and subjects gave their written informed consent.

Blood Samples and Analysis of Lymphocyte Subsets

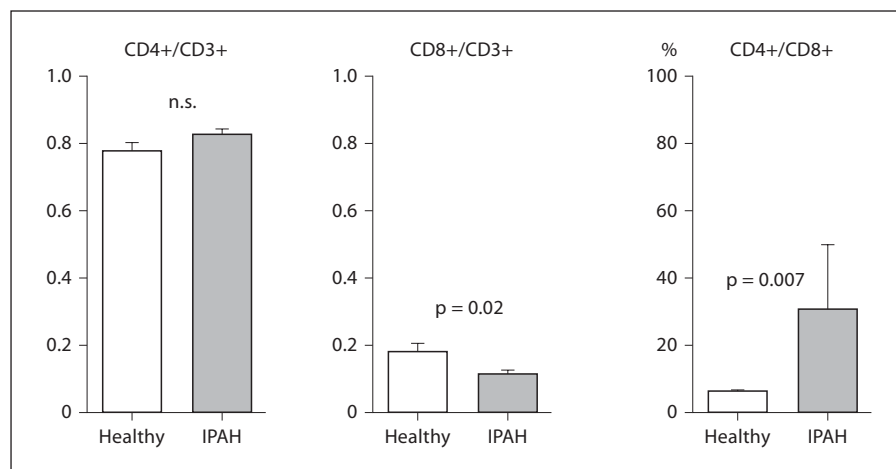
Venous blood samples were collected in heparin-treated tubes from each subject, and peripheral blood mononuclear cells (PBMCs) were separated immediately using Histopaque (Sigma-Aldrich, St. Louis, Mo., USA) density gradient centrifugation. PBMCs were either processed within 24 h (18 patients and 23 controls) or frozen at -20°C (18 patients and 10 controls) for up to 1 month in PBMC freezing medium (Cambrex, Walkersville, Md., USA).

The FACS Calibur 4-color flow cytometer (BD Biosciences, N.Y., USA) was used to identify the cell type. Two million PBMCs each were stained with fluorescently labeled monoclonal antibodies (mAbs) directed against superficial activation markers (anti-CD3-PE, anti-CD8-FITC, anti-CD4-perCP, anti-CD25-APC) and against the intranuclear marker anti-FoxP3-PE. The superficial markers were all purchased from BD Bioscience, whereas the intracellular marker was purchased from eBioscience (San Diego, Calif., USA). In brief, for superficial stains, cells were incubated in the dark at -4°C for 30 min with mAbs at concentrations recommended by the manufacturer, washed twice with phosphate-buffered saline, blocked with 1% bovine serum albumin and thereafter fixed in phosphate-buffered saline with 1% formaldehyde and 0.01% NaN_3 and stored at -4°C in the dark until analysis. For intracellular staining with anti-FoxP3, cells were stained against the superficial markers CD4 and CD25 as above. After 1 wash, cells were permeabilized, washed, blocked and stained using the buffers and instructions provided by the manufacturer (eBioscience) and thereafter fixed and stored as above. Flow cytometric analysis was performed within 24 h. The flow cytometry data were analyzed using CellQuest software (BD Bioscience).

Cell Proliferation Assay

PBMCs were obtained from patients and controls as described above. CD4+CD25hi+ (T_{reg}) and CD4+CD25hi- (responders) T cells were separated from PBMCs using magnetic separation by RoboSep (StemCell, Vancouver, Canada). Cell purity was assessed by flow cytometry using fluorescently labeled anti-CD4-perCP and anti-CD25-APC mAbs. Before seeding, U-bottom 96-well plates were coated with anti-CD3 (0.5 $\mu\text{g}/\text{ml}$ for 4–16 h at -4°C). To measure cell proliferation, 1×10^4 CD4+CD25hi- T cells/well were seeded, and then increasing numbers (1:0, 1:8, 1:4, 1:2) of CD4+CD25hi+ (T_{reg}) cells were added and cocultured for 48 h. Cells were cultured in a final volume of 200 μl PBMC cell culture medium consisting of RPMI 1640 with glutamine containing 10%

Fig. 1. Differential frequencies of CD4+ and CD8+ T lymphocyte (CD3+) subsets for IPAH patients and controls. n.s. = Not significant.



human AB serum, 1% HEPES 1 M, 1% penicillin-streptomycin-glutamine (Cambrex, Ariz., USA) and 1% Na pyruvate 100 M at 37°C in 5% CO₂ for 48 h. Thereafter, cell plates were centrifuged at 1,400 rpm for 8 min. Cell proliferation was measured using CyQuant proliferation kit (Invitrogen, Molecular Probes, N.Y., USA).

Data Presentation and Statistics

Baseline characteristics and lymphocyte counts are presented as means \pm standard deviation. SPSS version 11.5 and GraphPad Prism version 4.0 software packages were used for statistical analysis and graph editing. Mann-Whitney U, Student's t and Wilcoxon tests were used for comparative analysis between groups as appropriate. A p value <0.05 was considered statistically significant.

Results

Patient Characteristics

We enrolled 36 patients (26 females) and 33 healthy controls (15 females) at a mean age of 51 ± 11 and 43 ± 9 years ($p = 0.05$) in the study upon informed consent. Pulmonary hemodynamic characteristics of the patients were: mean pulmonary arterial pressure 49 ± 14 mm Hg, cardiac index 2.3 ± 0.7 l \cdot min⁻¹ \cdot m⁻², pulmonary vascular resistance 858 ± 364 dyn \cdot s \cdot m⁻⁵, pulmonary capillary occlusion pressure 8.9 ± 4.1 mm Hg, right arterial pressure 8.1 ± 5.4 mm Hg, arterial oxygen saturation $93 \pm 6\%$, and mixed venous oxygen saturation $54 \pm 9\%$. The mean time between the diagnostic right heart catheter date and the blood analysis was 10 ± 10 months. Twenty-five patients (68%) were treated with continuous intravenous epoprostenol, 3 of which had a combination therapy with oral bosentan. Two pa-

tients (5%) were treated with inhaled iloprost and 1 patient with sildenafil. Eight patients (22%) did not receive a specific therapy for PAH.

Differential Frequencies of Lymphocyte Subclasses and T_{reg} Cells

Within the T cell subset, we found a tendency toward an increase in the percentage of CD4+ T cells in the peripheral blood of IPAH versus controls (83 ± 1.7 vs. $78 \pm 2.7\%$; $p = 0.11$) at the expense of a significant decrease in CD8+ T cells (11 ± 1.2 vs. $18 \pm 2.5\%$; $p = 0.02$) (fig. 1). The CD4/CD8 relation consecutively decreased ($p = 0.007$) (fig. 1). Within the CD4+ T cell compartment, we found significantly more CD25hi+ T cells in patients compared with controls (5.9 ± 0.8 vs. $3.5 \pm 0.3\%$; $p = 0.009$) and a significantly increased number of FoxP3+ T_{reg} cells (10.1 ± 1.5 vs. $4.5 \pm 0.35\%$ of all CD4+ T cells; $p < 0.001$) (fig. 2, 3). However, the percentage of FoxP3+ cells within the CD25hi+CD4+ T_{reg} cells in each group was similar (61.6 ± 2.8 vs. $62.0 \pm 4.0\%$; $p = 0.93$). We found no difference in the lymphocyte subsets between patients who received PAH-specific therapy and therapy-naïve patients. We neither found a difference in the lymphocyte subsets between patients with a time span between the diagnostic pulmonary artery catheter of more and less than 10 months.

Proliferation Assay

The proliferation assay of T lymphocytes from the blood of IPAH patients ($n = 16$) and healthy controls ($n = 13$) was performed in triplicate. Test performance was monitored by a respective linear rise of relative light units with increasing standard number of cells/well. Patient

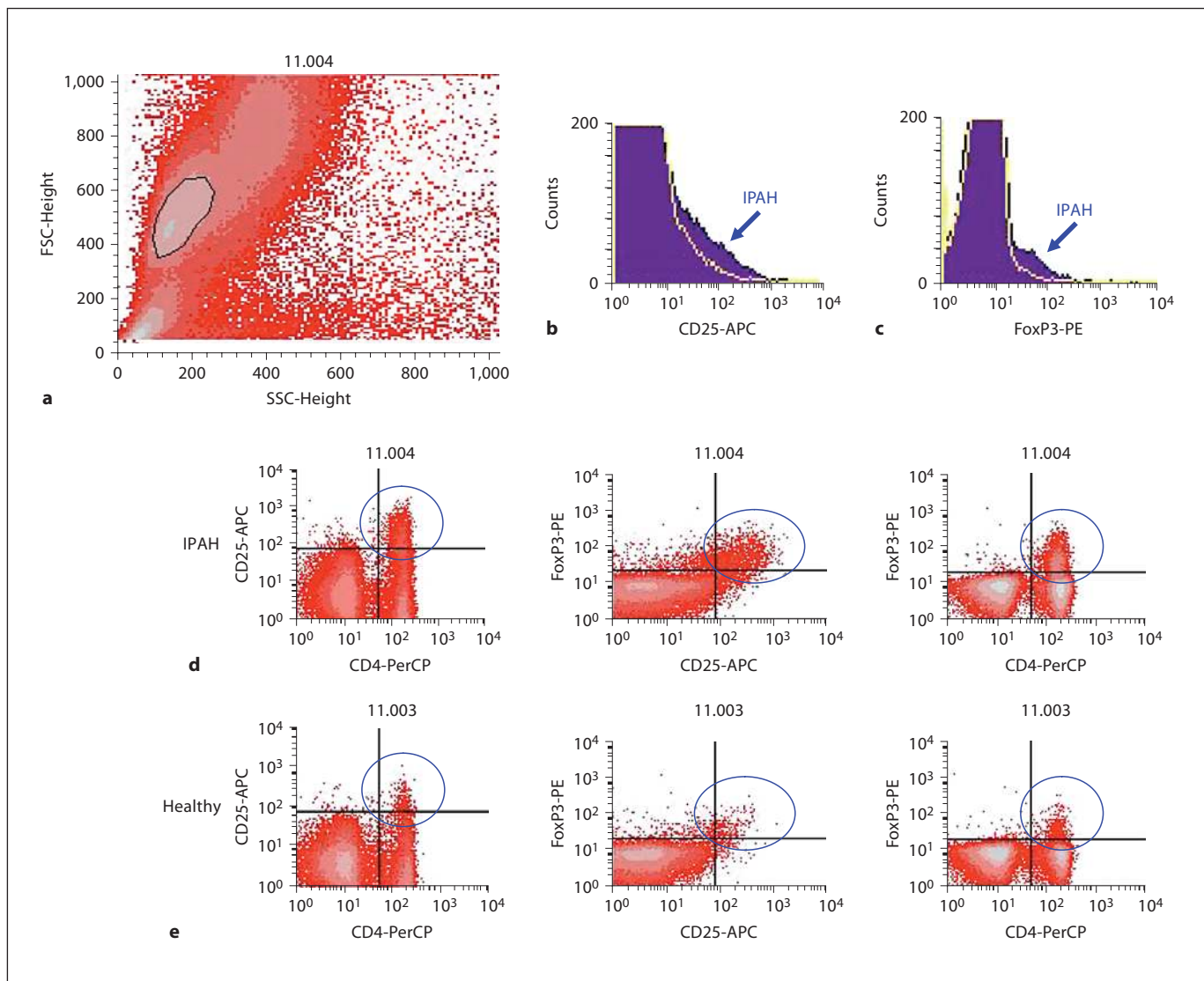


Fig. 2. Representative samples of flow cytometric findings are shown for a patient with IPAH and a healthy control. **a** A typical forward side scatter with the gated lymphocytes encircled in black. **b, c** Typical histograms of CD25+ (**b**) and FoxP3+ (**c**) T cells

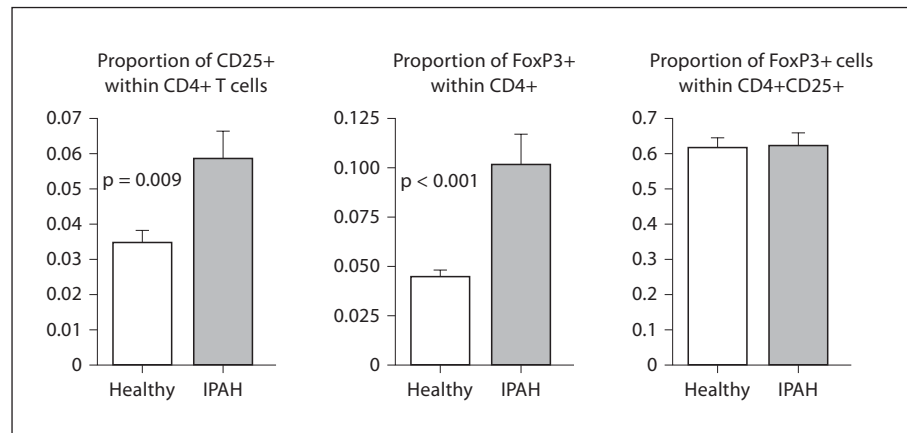
from IPAH patients, indicating also the typical histogram in healthy controls (Ctrl). **d, e** Four field charts show typical cell distributions for the markers of interest (CD4, CD25 and FoxP3) for IPAH patients (**d**) and healthy controls (**e**).

CD4+CD25[−] T cells (responder cells) were found to have proliferated slightly more than cells from healthy controls ($9,731 \pm 2,638$ vs. $7,715 \pm 1,310$ relative light units); however, the difference was not statistically significant ($p = 0.6$). Addition of increasing numbers of CD4+CD25⁺ T cells equally suppressed the anti-CD3-induced proliferation of CD4+CD25[−] T cells in patients or controls (data not shown).

Discussion

In the present study, we found an increased percentage of CD25^{high}+ and FoxP3+CD4+ T_{reg} cells and a decreased percentage of CD8+ T cells in the peripheral blood of patients with IPAH compared with healthy controls. The percentage of FoxP3+ T cells within the CD25+CD4+ T cell subset of IPAH and controls was comparable, which still signifies a higher total percentage of FoxP3+CD25+ T_{reg} cells in IPAH due to their higher

Fig. 3. Differential frequencies of lymphocyte subclasses for IPAH patients and controls for CD25+ and FoxP3+CD4+ and FoxP3+CD25+CD4+ T cells. p values indicate the level of significance of the difference (2-sided t test).



percentage of CD25+ T cells (fig. 3). Our findings of a differential prevalence of some of the T cell subsets between IPAH and controls may point toward a pathogenetic role of the T cell compartment in PAH, as has recently been demonstrated in an animal model [27]. However, we are aware that the interpretation of our findings in the peripheral blood of IPAH compared with controls is difficult and does not allow to draw firm conclusions about pathogenetic pathways, causes and consequences in the pulmonary vasculature. Nevertheless, we present some speculations below in the hope to inspire future research in the field.

The transcription factor FoxP3, which is expressed by the majority of CD25+CD4+ and also by a fraction of CD25–CD4+ T cells, is the most reliable and specific marker of naturally occurring T_{reg} cells [35, 37]. The overall increase in FoxP3+CD4+ T cells in the blood of IPAH with an unchanged percentage of FoxP3+ cells within the CD25+CD4+ T cell subset indicates that at least some of the increased number of the FoxP3+ T cells can be attributed to a peripheral conversion of CD25–CD4+ T cells into T_{reg} cells [53]. Conversion of CD4+ T cells in the periphery into T_{reg} cells by de novo FoxP3+ expression has been described in various diseases in order to control an exaggerated immune response [36, 37, 53, 54]. Recent reports underscore that T_{reg} cells not only play a central role in the maintenance of immunotolerance [30, 36–38, 54], but that they are also potent inhibitors of antitumor and possibly antiviral immune responses [25, 42–45]. Monoclonal cancer-like endothelial proliferation is a key histopathological finding in IPAH [1, 3, 23, 55].

Therefore, we speculate that the increase in T_{reg} cells in the peripheral blood of IPAH could be attributed to the necessity to suppress any self-reactive T cells (possibly to-

wards the cancer-like endothelial proliferation) in analogy to the postulated function of increased T_{reg} cells in cancer [56, 57]. Tumor immune surveillance by T_{reg} cells is apparently insufficient to suppress the emergence of cancer. A possible clue to this issue is the finding that many tumor antigens are recognized by autologous cytotoxic T cells directed against normal self-constituents [57], thus indicating that tumor immunity is to some degree also autoimmunity. Whether cytotoxic T cells directed towards self-constituents might be involved in the pathogenesis of PAH is not known to date, but several findings already point toward a role of autoimmunity in PAH [8]. Our findings of increased T_{reg} cells in IPAH support this hypothesis. A functional hallmark of naturally occurring T_{reg} cells is that they suppress the activation and expansion of self-reactive T cells and inhibit the development of autoimmune disease [37]. The high sensitivity of T_{reg} cells toward self- and non-self antigens and their high activity of bystander suppression of other T cells suits their role of sustaining self-tolerance, but potentially hinders tumor immunity or the proper work of the acquired immune system in viral defense. Another speculation is that the peripheral conversion of CD4+ T cells into T_{reg} cells could be induced by microbial, particularly viral, infection. The association of some chronic viral infections with pulmonary hypertension is well known [52, 58, 59], and some findings point toward a potential role of microbial (mainly viral) antigen mimicry in the pathogenesis of IPAH [60], although to date, no specific microbial antigen could be consistently demonstrated [61, 62]. Besides an increase in T_{reg} cells, in the present study, we also found a significant decrease in CD8+ T cells. A decreased number or impaired function of CD8+ T cells (so called exhausted T cells) has been described in chronic viral infections [63–

66]. It is conceivable that a yet unknown factor in chronic viral infections of the pulmonary vasculature in PAH would trigger a depletion of cytotoxic T cells along with an increase in T_{reg} cells. Moreover, such a viral infection might also provoke the expression of epitopes on immune or endothelial cells against which an altered immune response in PAH might be directed. Future studies with a focus on the detection of viral or self antigens in the pulmonary vasculature against which the altered T cell subsets are directed would be desirable.

Plexiform lesions in lung tissue of patients with pulmonary hypertension are not only surrounded by lymphocytes and mast cells [21, 67], but also by dendritic cells (DCs), as has been described recently [68]. DCs play a role in immune regulation as professional antigen-presenting cells and controllers of the differentiation of naïve T cells into T_{reg} cells [69, 70]. Whereas inflammatory stimuli induce DC maturation, which direct naïve T cells to differentiate into effector T cells, immature DCs were only recently acknowledged to have an important role in the maintenance of immune tolerance through induction of T_{reg} cells [69, 71, 72]. On the other hand, T_{reg} cells talk back to DCs restraining their maturation and antigen presentation [73]. Therefore, it could be hypothesized that our finding and the finding of Perros et al. [68] point towards a mutual interaction between T_{reg} cells and DCs in order to keep up a state of tolerance. It remains to be questioned at which time point in the course of the disease such a state of tolerance develops, e.g., it could be imagined that an autoimmune constellation early in the disease course would later on develop into a state of tolerance with increased T_{reg} cells and DCs. However, in our study population, we found no difference between lymphocyte subsets obtained from patients within 10 months of the initial right heart catheterization and lymphocyte subsets obtained from patients with a time span of more than 10 month from diagnosis, although our study was not designed to look at changes of lymphocyte subsets over time.

The association of PAH with various autoimmune disorders is well known [8, 9, 24, 74–78], and findings such as anti-endothelial cell or anti-fibroblast antibodies in patients with IPAH or scleroderma-associated PAH suggest a pathogenetic role of autoimmunity in PAH [19, 20]. As many autoimmune diseases have been found to be associated with decreased T_{reg} cells [36, 40, 46, 49, 79], our findings of increased T_{reg} cells might be unexpected. Possible explanations could be that T_{reg} cells in the peripheral blood would be reactively elevated, along with a possible decrease in T_{reg} cells early in the disease course, or that, as discussed above, the increased T_{reg} cells represent

some state of immune tolerance in the course of the disease, possibly induced by a tumor or infectious antigen, leading to cancer-like proliferations in the pulmonary vasculature [46, 55].

Another possibility would be that the difference in lymphocyte subsets between our patients and controls could be attributed to simple confounders like age, gender or drug use. Patients were on average slightly older and more likely to be female than controls. In normal humans, total lymphocyte count, CD8+ T and CD19+ B cell subsets have been described to decrease beyond the age of 50 years, whereas female sex is related to higher CD4+ T cells [80], data on T_{reg} cells is lacking. As our patients were on average slightly older than 50 years (51 ± 11), it cannot be excluded that some of the differences we found between patients and controls could be attributed to age.

Drug use might alter T_{reg} cell number and possibly activity as well. Steroids have been found to increase T_{reg} cells [81, 82]; therefore, we included only steroid-naïve patients. Nevertheless, specific drugs used in the treatment of PAH (such as prostanoids, endothelin receptor antagonists or phosphodiesterase inhibitors) have a potential to influence immune regulation and thereby lymphocyte subsets [83–86]. Sixty-eight percent of the patients in our cohort were on continuous intravenous epoprostenol therapy. All the same, we did not find a difference in lymphocyte subsets between treated and untreated patients. However, this study was not designed to look for effects of therapy on lymphocyte subsets; therefore, it could still be possible that some of the observed differences between patients and controls can be attributed to therapy. Future studies are warranted to answer questions about the influence of therapy on lymphocyte subsets.

In summary, we believe that our findings of increased T_{reg} cells and decreased CD8+ T cells in the peripheral blood of patients with IPAH are important and may have significant implications in directing future research in the field. Whether alterations in lymphocyte subsets actually play a role in the pathobiology of PAH and whether they are a cause or a consequence remains to be investigated.

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